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Purification and characterization of an allosteric fructose-1,6-bisphosphate aldolase from germinating mung beans (*Vigna radiata*)

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Abstract

Cytosolic fructose-1,6-P_2 (FBP) aldolase (ALD_c) from germinated mung beans has been purified 1078-fold to electrophoretic homogeneity and a final specific activity of 15.1 μmol FBP cleaved/min per mg of protein. SDS–PAGE of the final preparation revealed a single protein-staining band of 40 kDa that cross-reacted strongly with rabbit anti-(carrot ALD_c)-IgG. The enzyme's native M_r was determined by gel filtration chromatography to be 160 kDa, indicating a homotetrameric quaternary structure. This ALD is a class I ALD, since EDTA or Mg^{2+} had no effect on its activity, and was relatively heat-stable losing 0–25% of its activity when incubated for 5 min at 55–65 °C. It demonstrated: (i) a temperature coefficient (Q_{10}) of 1.7; (ii) an activation energy of 9.2 kcal/mol active site; and (iii) a broad pH-activity optima of 7.5. Mung bean ALD_c is bifunctional for FBP and sedoheptulose-1,7-P_2 ($K_m \approx 17$ μM for both substrates). ATP, ADP, AMP and ribose-5-*P* exerted inhibitory effects on the activity of the purified enzyme. Ribose-5-*P*, ADP and AMP functioned as competitive inhibitors (K_i values = 2.2, 3.1 and 7.5 mM, respectively). By contrast, the addition of 2 mM ATP: (i) reduced V_{max} by about 2-fold, (ii) increased K_m(FBP) by about 4-fold, and (iii) shifted the FBP saturation kinetic plot from hyperbolic to sigmoidal ($h = 1.0$ and 2.6 in the absence and presence of 2 mM ATP, respectively). Potent feedback inhibition of ALD_c by ATP is suggested to help balance cellular ATP demands with the control of cytosolic glycolysis and respiration in germinating mung beans.

Keywords: Aldolase; Plant glycolysis; Metabolic control; Carbohydrate metabolism (seed); *Vigna radiata*

1. Introduction

Fructose-1,6-bisphosphate (FBP[2]) aldolase (ALD; D-Fru-1,6-bisphosphate D-glyceraldehyde-3-P-lyase; E.C. 4.1.2.13) is a ubiquitous and abundant glycolytic enzyme that catalyzes the reversible aldol cleavage of FBP into glyceraldehyde-3-*P* and dihydroxyacetone-*P*. ALDs are either dependent upon divalent cations for activity (Class II ALD) or not (Class I ALD). Class II ALDs occur in fungi and prokaryotes and are homodimers with M_rs of about 80 kDa, whereas the enzymes from animals and vascular plants fall into Class I and are homotetramers (M_r approx. 160 kDa). In mammals, three tissue-specific ALD isozymes have been identified in muscle, liver or kidney, and brain (Gefflaut et al., 1995). These isozymes have slightly different physical features, and the muscle and liver ALDs also vary in their substrate specificities.

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[2] *Abbreviations:* ALD, fructose-1,6-P_2 aldolase; ALD_c and ALD_p, cytosolic and plastidic isozymes of fructose-1,6-P_2 aldolase, respectively; FBP, fructose-1,6-P_2; FPLC, fast protein liquid chromatography; SBP, sedoheptulose-1,7-P_2.

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The occurrence of tissue-specific ALD isozymes in vascular plants has not yet been established. However, cytosolic and plastidic ALD isozymes (ALD_c and ALD_p, respectively) are expressed in photosynthetic and non-photosynthetic plant tissues and are encoded by separate nuclear genes that likely evolved from duplication of a common ancestral gene (Plaxton, 1996). ALD_c and ALD_p differ somewhat in their respective thermal stability, net charge, amino acid composition, immunological properties and subunit size. In photosynthetic tissues ALD_p plays a pivotal role in the C_3 reductive pentose-*P* (Calvin–Benson) cycle and represents as much as 95% of the total leaf ALD activity (Krüger and Schnarrenberger, 1983; Lebherz et al., 1984). Purified ALD_p and ALD_c preparations from various leaves have been characterized (Krüger and Schnarrenberger, 1983; Lebherz et al., 1984; Marsh et al., 1989; Schnarrenberger and Krüger, 1986). Comparatively little is known about the ALDs from non-photosynthetic plant tissues. However, the contribution of ALD_c to the total ALD activity of non-green tissues appears to be much greater than in green leaves and often accounts for more than 50% of the total ALD activity present (Krüger and Schnarrenberger, 1985; Nishimura and Beevers, 1981; Botha and O'Kennedy, 1989; Moorhead and Plaxton, 1990; Hodgson and Plaxton, 1998; Schwab et al., 2001). The physical, immunological and kinetic properties of homogeneous ALD_c preparations from non-green plant tissues have thus far only been reported for the germinating *Phaseolus vulgaris* and *Ricinus communis* seed and carrot storage root enzymes (Botha and O'Kennedy, 1989; Moorhead and Plaxton, 1990; Hodgson and Plaxton, 1998).

It is generally believed that a key glycolytic control site is the conversion of Fructose-6-*P* to FBP, a reaction catalyzed in the plant cytosol by the ATP- or PPi-dependent phosphofructokinase (Plaxton, 1996). Although ALD catalyzes a readily reversible reaction and is not generally considered to be an important regulatory enzyme, it may exert significant metabolic control in vivo since: (i) moderate reductions in ALD_p activity by antisense techniques markedly inhibited photosynthetic CO_2 fixation and growth of transgenic potato plants (Haake et al., 1998), and (ii) the in vitro activity of several purified plant and animal ALDs is significantly modulated by physiologically relevant concentrations of various metabolite effectors (Botha and O'Kennedy, 1989; Moorhead and Plaxton, 1990; Hodgson and Plaxton, 1998; Akkerman, 1985; MacDonald and Storey, 2002). Furthermore, ALD_c has been suggested to physically interact in vivo with other functionally related glycolytic or gluconeogenic enzymes, including cytosolic ATP- and PPi-dependent phosphofructokinases in carrot storage roots (Moorhead and Plaxton, 1992), as well as cytosolic FBPase during gluconeogenesis in the mammalian liver and germinating *R. communis* endosperm (Moorhead et al., 1994; Pontremoli et al., 1979). ALD_c was one of seven different glycolytic enzymes reported to be physically associated with the cytosolic face of the outer membrane of mitochondria isolated from *Arabidopsis thaliana* suspension cells (Giegé et al., 2003). The enzyme has also been implicated as a key 'scaffolding' enzyme during the formation of a glycolytic complex on actin-containing filaments in contracting mammalian muscle tissues (Ovádi and Srere, 2000). In the present study we report the purification of ALD_c from germinated mung beans and characterize the enzyme's physical, immunological and kinetic properties. Potent allosteric inhibition of the enzyme by ATP is suggested to contribute to the fine metabolic control of glycolysis and respiration in this tissue.

2. Result and discussion

2.1. Enzyme purification

The specific ALD activity in clarified extracts of germinated mung beans gradually increased to a maximum in 30 h to about 0.014 U/mg, following imbibition. Thereafter, ALD specific activity showed a marked decline (results not shown). A similar developmental profile has been noted for several other glycolytic enzymes of germinated mung beans (Kumar and Malhotra, 1992; Malhotra and Kayastha, 1990; Malhotra et al., 1979). As outlined in Table 1, ALD from 30 h germinated mung beans was purified 1078-fold to a final specific activity of 15.1 U/mg and an overall yield of 21%.

About 35% of the total ALD activity applied to the phosphocellulose column consistently failed to absorb to this matrix, even when the resin bed volume was increased by 200%. Similarly, ALD_p, but not ALD_c, from spinach leaves (Krüger and Schnarrenberger, 1983) and germinated *P. vulgaris* seeds (Botha and O'Kennedy, 1989) do not bind to phosphocellulose, thereby enabling easy separation from the corresponding ALD_c.

2.2. Physical and immunological properties

SDS–PAGE of the final ALD preparation revealed a single protein-staining polypeptide of about 40 kDa that strongly cross-reacted with rabbit anti-(carrot ALD_c)-IgG (Fig. 1A and B). Plant and animal ALD_cs exhibit an identical subunit M_r of 40 kDa, whereas vascular plant ALD_p has a slightly smaller subunit M_r of about 38 kDa (Lebherz et al., 1984; Botha and O'Kennedy, 1989; Moorhead and Plaxton, 1990; Hodgson and Plaxton, 1998; Schwab et al., 2001). Homogeneity of the final preparation was confirmed by non-denaturing PAGE which generated a single protein-staining polypeptide that co-migrated with ALD activity (Fig. 2). No cross-reactivity was observed when an immunoblot of the

Table 1
Purification of cytosolic aldolase from 100 g of 30-h germinated mung beans

Step	Volume (ml)	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Clarified extract	120	35	2510	0.014	–	–
Acid step (pH 5.5)	110	35	1000	0.035	2.5	100
$(NH_4)_2SO_4$ fractionation	7	18.5	120	0.15	11	53
Phosphocellulose chromatography	12	8.8	0.70	12.6	893	25
DEAE-cellulose chromatography[a]	0.5	7.4	0.49	15.1	1071	21

[a] Concentrated pooled fractions.

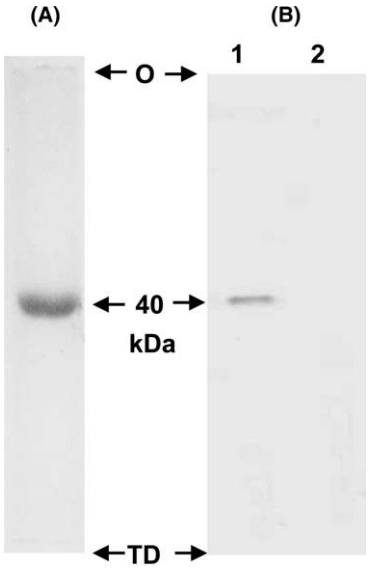

Fig. 1. SDS–PAGE and immunoblot analysis of ALD_c from germinated mung beans. (A) SDS–PAGE (12% separating gel) of 5 μg of purified mung bean ALD_c stained with Coomassie Blue R-250. (B) Immunoblot analysis was performed using affinity-purified rabbit anti-(carrot storage root ALD_c)-IgG (Moorhead and Plaxton, 1990). Immunoreactive polypeptides were detected using an alkaline phosphatase-conjugated secondary antibody; phosphatase staining was for 4 min at 30 °C. Lane 1 contains 50 ng of purified mung bean ALD_c, whereas lane 2 contains 7.5 μg of the unbound protein fraction eluting from the phosphocellulose column. The indicated M_r is based upon the mobility of SDS–PAGE protein standards. O, origin; TD, tracking dye front.

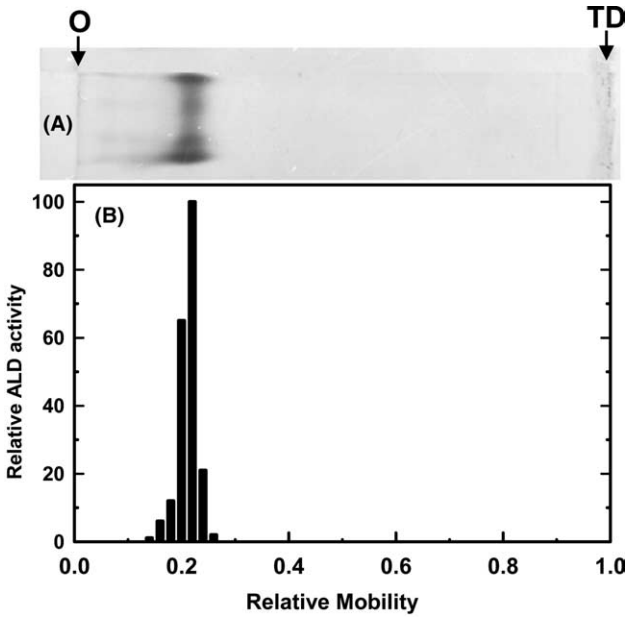

Fig. 2. Non-denaturing PAGE of purified mung bean ALD_c. The electrophoresis was carried out in a 7.5% (w/v) resolving gel. (A) Protein staining was performed with Coomassie Blue R-250. (B) Distribution of ALD activity; activity was determined as described in the Materials and Methods. The protein-stained lane contains 3 μg of protein, whereas the lane used to detect ALD activity contains 7 μg of protein. O, origin; TD, tracking dye front.

unbound protein fraction eluting from the phosphocellulose column was probed with the anti-(carrot ALD_c)-IgG (Fig. 1B, lane 2). The same anti-(carrot ALD_c)-IgG specifically cross-reacted with ALD_c, but not ALD_p, from spinach leaves (Moorhead and Plaxton, 1990).

The purified ALD was relatively heat stable, losing 0%, 10% and 25% of its activity when incubated for 5 min at 55, 60 and 65 °C, respectively. Heat stability appears to be a characteristic shared by all plant ALD_cs examined to date (Lebherz et al., 1984; Botha and O'Kennedy, 1989; Moorhead and Plaxton, 1990; Hodgson and Plaxton, 1998; Schwab et al., 2001). By contrast, the heat-labile plant ALD_p is completely inactivated by similar heat treatments (Lebherz et al., 1984; Schwab et al., 2001). Taken together, the aforementioned results demonstrate that the purified mung bean ALD corresponds to ALD_c rather than ALD_p.

The native M_r of mung bean ALD_c was estimated to be 160 kDa by gel filtration FPLC of the final preparation on a calibrated Superose 6 HR 10/30 column. Thus, as with all other plant and animal ALD_cs studied to date, the native mung bean enzyme appears to exist as a homotetramer.

2.3. Kinetic properties

2.3.1. Effect of temperature

The temperature coefficient (Q_{10}) for the reaction catalyzed by mung bean ALD_c was 1.7. An Arrhenius plot was linear over the range 4–40 °C (Fig. 3). The activation energy (E_a) for the enzyme was calculated from the Arrhenius equation to be 9.2 kcal/mol active site.

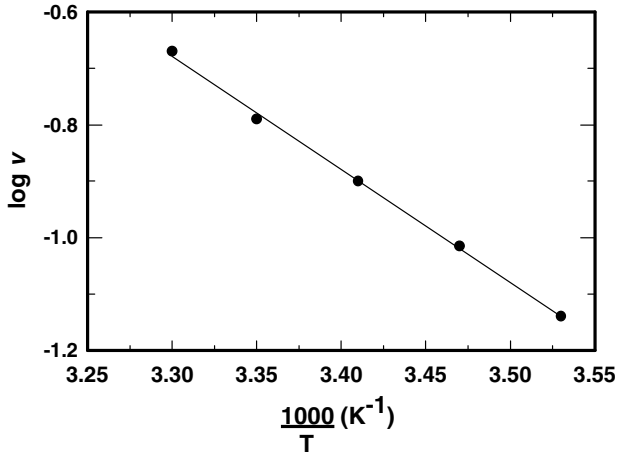

Fig. 3. Arrhenius plot ($\log v$ versus $1000/T$) for the effect of temperature on aldolase activity. Assay conditions are described in the experimental section. The rate of the reaction (v) has been expressed as the rate of absorbance decrease per min.

2.3.2. Effect of pH

Similar to ALD_cs from other plant tissues (Lebherz et al., 1984; Schnarrenberger and Krüger, 1986; Botha and O'Kennedy, 1989; Moorhead and Plaxton, 1990; Hodgson and Plaxton, 1998; Schwab et al., 2001) the purified ALD_c demonstrated a broad pH/activity profile with a maximum occurring at about pH 7.5 (results not shown). All subsequent kinetic studies were carried out at pH 7.5.

2.4. Effect of cations

ALD_c activity was assayed in the absence and presence of 1 mM $MgCl_2$ or 1 mM EDTA. No difference in activity was detected with or without $MgCl_2$ or EDTA. Thus, as is the case with ALDs from other eukaryotic sources, the mung bean ALD_c is a class I ALD (non-metal-requiring).

2.5. Substrate specificity

Similar to other plant ALD_cs (Botha and O'Kennedy, 1989; Moorhead and Plaxton, 1990; Hodgson and Plaxton, 1998) the mung bean enzyme catalyzed the aldol cleavage of FBP and SBP. Table 2 shows the V_{max}, k_{cat}, K_m, and specificity constants (k_{cat}/K_m) obtained with FBP and SBP as substrates. Notable are the relatively low catalytic-centre activities for FBP and SBP, respectively (Table 2). In both cases, Michaelis–Menten saturation kinetics were observed. Although the K_m values were equivalent, the V_{max}, k_{cat}, and corresponding specificity constant values with FBP were slightly (20%) greater than those obtained with SBP. In contrast, plant ALD_p generally exhibit significantly lower K_m(SBP) values than their corresponding K_m(FBP) values (Krüger and Schnarrenberger, 1983; Flechner et al., 1999). Our

Table 2
Kinetic parameters for substrates of cytosolic aldolase from germinated mung beans

Substrate	V_{max} (μmol substrate cleaved/min/mg protein)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})
FBP	15	40	16.7	2.4
SBP	12.5	33.3	16.7	2.0

All values are the mean of at least three independent determinations and are reproducible to within ±10% (SE) of the mean value.

results imply that ALD_c might play a bifunctional role in catalyzing the aldol cleavage of FBP and SBP in the cytosol of the germinated mung bean. While the bifunctional role of class I ALD_p for the reversible FBP and SBP condensation in the reductive pentose-*P* cycle of vascular plants is well known (Flechner et al., 1999), this has yet to be firmly established for plant ALD_c. Neither fructose-1-*P* nor fructose-6-*P* (10 mM each) served as substrates for mung bean ALD_c. This was also reported for purified carrot ALD_c (Moorhead and Plaxton, 1990), but differs from pea, wheat, and corn leaf ALD_cs, as well as castor bean ALD_c, which all showed activity at high fructose-1-*P* concentrations (Schnarrenberger and Krüger, 1986; Hodgson and Plaxton, 1998).

2.6. Metabolite effectors

A variety of compounds were tested for effects on mung bean ALD_cactivity at subsaturating (20 μM) FBP. The following substances had no effect (±10% control rate) on enzyme activity: dithiothreitol, Pi, *P*-enolpyruvate, 3-*P*-gluconate, citrate, glucose, glucose-1-*P*, glucose-6-*P*, fructose, ribose, arabinose, adenosine and sucrose (5 mM each); and fructose-2,6-P_2 (50 μM). None of the compounds tested served as activators. However, ribose-5-*P*, AMP, ADP, and particularly ATP were effective inhibitors (Table 3). Inhibition by ribose-5-*P*, ADP and AMP was further evaluated and uniformly determined to be competitive (Fig. 4) with corresponding K_i values of 2.2, 3.1 and 7.5 mM, respectively.

Notably, the presence of physiological concentrations of ATP (2 mM) (Kobr and Beevers, 1971; Podestá and Plaxton, 1991; Siegnthaler and Douet-Orhant, 1994) markedly influenced FBP binding by the enzyme such that V_{max} was lowered by about 2-fold, K_m(FBP) increased by about 4-fold, and the FBP saturation plot shifted from hyperbolic to sigmoidal (Fig. 5). This was not an artifact of the coupled assay system since the addition of extra coupling enzymes (5 U mL^{-1} each of triose-*P* isomerase and glycerol-3-*P* dehydrogenase) or 0.1 mM NADH at the completion of each assay had no influence on the degree of ALD_c inhibition that was observed in the presence of 2 mM ATP. Hill plots of the FBP saturation kinetic data obtained in the

Table 3
Influence of several metabolites on the activity of purified ALD_c from germinated mung beans

Addition	Relative activity (%)
5 mM Ribose-5-*P*	49
5 mM AMP	76
5 mM ADP	60
2 mM ATP	6

Standard assay conditions were used except that the FBP concentration was subsaturating (20 μM). Activities are expressed relative to the control determined in the absence of any additions and set at 100%. All values are the mean of at least 3 independent determinations and are reproducible to within ±10% (SE) of the mean value.

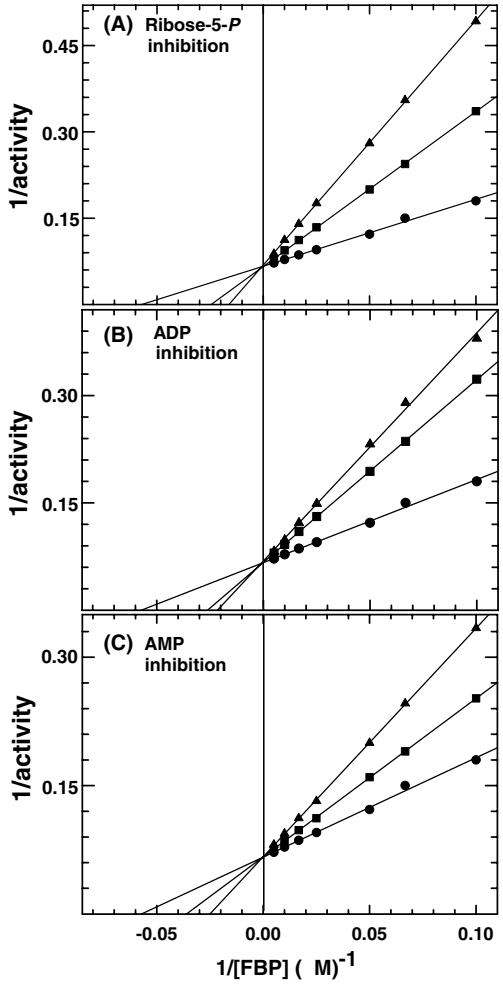

Fig. 4. Inhibition of mung bean ALDc by ribose-5-P (A), ADP (B), and AMP (C). Enzyme activity was determined at varying concentrations of FBP in the presence of 0 mM (●), 3 mM (■) or 6 mM (▲) in case of (A); 0 mM (●), 4 mM (■) or 6 mM (▲) in case of (B) and 0 mM (●), 5 mM (■) or 10 mM (▲) in case of (C). Activity is expressed as μmol FBP cleaved min^{-1} mg^{-1} protein.

presence and absence of 2 mM ATP are shown in Fig. 5 (inset). Corresponding Hill coefficients (*h*) were determined to be 2.6 and 1.0 in the presence and absence of 2 mM ATP, respectively. These data indicate that the ATP binding promotes a conformational change causing the enzyme to exhibit positive cooperativity with respect to FBP binding. To the best of our knowledge this is the first time that any ALD has been demonstrated to exhibit sigmoidal FBP saturation kinetics. As ALD catalyzes a reaction that is close to equilibrium in vivo (Plaxton, 1996), the direction of flux through the enzyme will largely be dictated by FBP: triose-*P* mass action ratios. However, our results imply that ALD_c may participate in the control of glycolytic flux in the cytosol of germinated mung beans. Inhibition of the enzyme by elevated cytosolic ATP concentrations may exert significant respiratory control in vivo. Further studies are required to fully assess the potential role of ALD_c in the control of plant glycolysis and respiration.

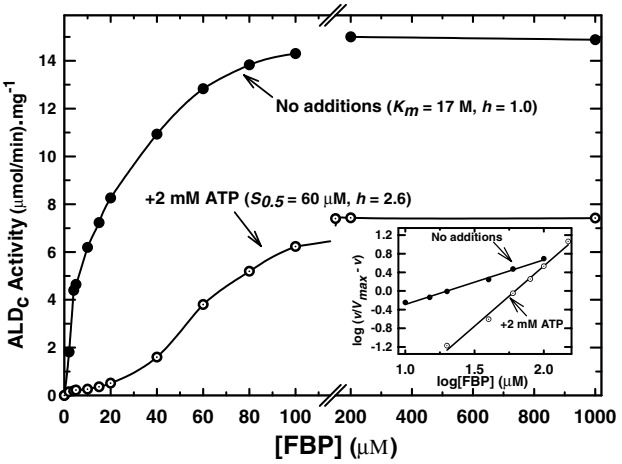

Fig. 5. Influence of ATP on the FBP saturation kinetics of purified ALD_c from germinated mung beans. The inset shows the Hill plot of the data.

3. Experimental

3.1. Chemicals and plant material

Biochemicals, coupling enzymes, and protein standards were purchased from Sigma–Aldrich Corporation. All other reagents were of analytical grade and were supplied by BDH Chemicals or Sisco Research Laboratories. All solutions were prepared using double distilled water.

Mung beans (*Vigna radiata*, cv. Wilczek) were generously provided by the Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University, and imbibed for 8 h in water at 25 °C, and germinated in the dark at 30 °C by spreading the imbibed seeds over moist filter paper on a moist sand bed. For enzyme purification, 30-h germinated seeds (containing radical and cotyledons) were used.

3.2. Enzyme and protein assays, and kinetic studies

The ALD reaction was routinely coupled with the triose-*P* isomerase and glycerol-3-*P* dehydrogenase

reactions and assayed in the forward (glycolytic) direction at 30 °C by continuously monitoring NADH utilization at 340 nm using a Spectronic 1001 spectrophotometer. Standard assay conditions for ALD were: 50 mM Tris–HCl (pH 7.5), 1 mM FBP, 0.1 mM NADH, 10 U triose-*P* isomerase and 1 U glycerol-3-*P* dehydrogenase in a final volume of 1 ml. Coupling enzymes were desalted before use. All assays were initiated by the addition of enzyme preparation and were corrected for NADH oxidase activity, when necessary, by omitting FBP from the reaction mixture. One unit of activity is defined as the amount of enzyme resulting in cleavage of 1 μmol substrate/min at 30 °C, equivalent to the oxidation of 2 μmol NADH in the case of FBP and of 1 μmol NADH in the case of sedoheptulose-1,7-P_2 (SBP). In all cases the rate of reaction was proportional to concentration of enzyme assayed and remained linear with respect to time. Apparent K_m, $S_{0.5}$, and h (Hill coefficient) values were determined from the Hill equation using Sigma Plot. Competitive inhibition constants (K_i values) were determined from Dixon plots. All parameters are the mean of triplicate determinations from three independent preparations of the purified enzyme and are reproducible to within ±10% SE of the mean value.

Protein concentration was estimated by the Lowry et al. (1951) procedure using Bovine Serum Albumin (BSA) as the standard protein. Specific activity is expressed as enzyme U/mg protein.

3.3. Buffers used in aldolase purification

Buffer A: 50 mM Tris–HCl (pH 8.5) containing 10 mM β-mercaptoethanol. Buffer B: 20 mM Tris–HCl (pH 7.3) containing 10 mM β-mercaptoethanol.

3.4. Enzyme purification

All purification procedures were carried out at 4 °C. Germinated mung beans (100 g) were homogenized using Waring blender in 1 volume of buffer A, filtered through four layers of cheesecloth, and centrifuged at 21,000*g* for 15 min at 4 °C.

3.4.1. Acidification

The clarified extract was adjusted to pH 5.5 with 7 M acetic acid, gently stirred for 5 min, and centrifuged at 21,000*g* for 5 min. The supernatant was adjusted to pH 7.5 with 15 M NH_4OH. This treatment removed pigmented material which otherwise interfered with subsequent purification steps.

3.4.2. Ammonium sulphate fractionation

Proteins precipitating in the range 37–50% (saturation) ammonium sulphate were collected by centrifugation, dissolved in buffer A, and dialyzed against buffer A for 2 h with four buffer changes (500 ml each).

3.4.3. Phosphocellulose chromatography

The dialysate was absorbed at 0.5 ml/min onto a column (1.5 × 21 cm) of phosphocellulose (Whatman P11) pre-equilibrated with buffer B. The column was washed until the A_{280} decreased to baseline, then ALD eluted as a single peak with buffer B containing 1 mM FBP (fraction size 3 ml).

3.4.4. DEAE-cellulose chromatography

Pooled peak phosphocellulose fractions were absorbed at 0.5 ml/min onto a column (1 × 7 cm) of DEAE-cellulose (Sigma–Aldrich Corporation) pre-equilibrated with buffer B. The column was washed with buffer B containing 50 mM KCl until the A_{280} decreased to baseline, and ALD eluted with buffer B containing 100 mM KCl (fraction size 1 ml). Pooled peak activity fractions were concentrated to about 0.5 ml by dialysis against solid sucrose, divided into 20 μl aliquots, and stored at −20 °C. The purified enzyme was stable for at least 3 weeks when stored at −20 °C.

3.5. Electrophoresis and immunoblotting

Non-denaturing and SDS–PAGE, subunit M_r estimations, and immunoblotting were performed using the Bio-Rad mini-gel apparatus as previously described (Moorhead and Plaxton, 1992; Hodgson and Plaxton, 1998). Immunological specificities were confirmed by performing immunoblots in which rabbit pre-immune serum was substituted for the affinity-purified rabbit anti-(carrot ALD_c)-IgG. To detect ALD activity following non-denaturing PAGE, a lane was sliced into 2 mm segments and each slice was incubated in 0.15 ml of 50 mM Tris–HCl (pH 7.5), containing 0.1 mM-NADH, 10 units of triose-*P* isomerase and 1 U of glycerol-3-*P* dehydrogenase (final volume 1 ml). Relative ALD activity was determined by monitoring the decrease in A_{340} following a 15 min incubation at 30 °C.

3.6. Estimation of native molecular mass

This was done by FPLC on a Superose 6 HR 10/30 column, using 100 μl sample volumes and 25 mM potassium phosphate (pH 7.5) containing 15% (v/v) glycerol, 200 mM KCl, and 10 mM β-mercaptoethanol as the column buffer. Fractions (0.3 ml) were collected with a flow rate of 0.2 ml/min and assayed for protein and/or ALD activity. The native M_r of ALD was determined from a plot of K_D (partition coefficient) versus log M_r for the following protein standards: BSA (66 kDa), rabbit muscle glyceraldehyde-3-*P* dehydrogenase (145 kDa), horse liver alcohol dehydrogenase (150 kDa), rabbit muscle aldolase (160 kDa) and catalase (232 kDa).

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References

Akkerman, J.W.N., 1985. More on metabolic control – does fructose-1,6-bisphosphate reflect phosphofructokinase activity. Trends Biochem. Sci. 10, 187–188.

Botha, F.C., O'Kennedy, M.M., 1989. Characterization of the cytosolic aldolase from germinating *Phaseolus vulgaris* seeds. J. Plant Physiol. 135, 433–438.

Flechner, A., Gross, W., Martin, W.F., Schnarrenberger, C., 1999. Chloroplast class I and class II aldolases are bifunctional for fructose-1,6-biphosphate and sedoheptulose-1,7-biphosphate cleavage in the Calvin cycle. FEBS Lett. 447, 200–202.

Gefflaut, T., Blonski, C., Perie, J., Willson, M., 1995. Class I aldolases: substrate specificity, mechanism, inhibitors and structural aspects. Prog. Biophys. Mol. Biol. 63, 301–340.

Giegé, P., Heazlewood, J.L., Tosessner-Tunali, U., Millar, A.H., Fernie, A.R., Leaver, C.J., Sweetlove, L.J., 2003. Enzymes of glycolysis are functionally associated with the mitochondrion in *Arabidopsis* cells. Plant Cell 15, 2140–2151.

Haake, V., Zrenner, R., Sonnewald, U., Stitt, M., 1998. A moderate decrease of plastid aldolase activity inhibits photosynthesis, alters the level of sugars and starch, and inhibits growth of potato plants. Plant J. 14, 147–157.

Hodgson, R.J., Plaxton, W.C., 1998. Purification and characterization of cytosolic fructose-1,6-bisphosphate aldolase from endosperm of germinated castor oil seeds. Arch. Biochem. Biophys. 355, 189–196.

Kobr, M., Beevers, H., 1971. Gluconeogensis in the castor bean endosperm. I. Changes in glycolytic intermediates. Plant Physiol. (USA) 47, 48–52.

Krüger, I., Schnarrenberger, C., 1983. Purification, subunit structure and immunological comparison of fructose-bisphosphate alolases from spinach and corn leaves. Eur. J. Biochem. 136, 101–106.

Krüger, I., Schnarrenberger, C., 1985. Development of cytosol and chloroplast aldolases during germination of spinach seeds. Planta 164, 109–134.

Kumar, A., Malhotra, O.P., 1992. Effect of divalent metal ions and adenine nucleotides on phosphoglycerate kinase of mung beans. Plant Physiol. Biochem. 30, 655–663.

Lebherz, H.G., Leadbetter, M.M., Bradshaw, R.A., 1984. Isolation and characterization of the cytosolic and chloroplasts forms of spinach leaf fructose diphosphate aldolase. J. Biol. Chem. 259, 1011–1017.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol regent. J. Biol. Chem. 193, 265–275.

MacDonald, J.A., Storey, K.B., 2002. Purification and characterization of fructose bisphosphate aldolase from the ground squirrel, *Spermophilus lateralis*: enzyme role in mammalian hibernation. Arch. Biochem. Biophys. 408, 279–285.

Malhotra, O.P., Kayastha, A.M., 1990. Isolation and characterization of a phospho*enol*pyruvate phosphatase from germinating mung beans (*Vigna radiata*). Plant Physiol. (USA) 93, 194–200.

Malhotra, O.P., Srivastava, D.K., Srinivasan, 1979. Isolation and characterization of an alkali metal ion sensitive NAD^+-dependent glyceraldehyde 3-phosphate dehydrogenase from germinating green gram (*Phaseolus aurieus*). Arch. Biochem. Biophys. 197, 302–309.

Marsh, J.J., Wilson, K.J., Lebherz, H.G., 1989. Structural similarities between spinach chloroplast and cytosolic class-I fructose-1,6-bisphosphate aldolase- Immunological and amino-terminal amino acid sequence analysis. Plant Physiol. (USA) 91, 1393–1401.

Moorhead, G.B.G., Hodgson, R.J., Plaxton, W.C., 1994. Copurification of cytosolic fructose-1,6-bisphosphatase and cytosolic aldolase from endosperm of germinating castor oil seeds. Arch. Biochem. Biophys. 312, 326–335.

Moorhead, G.B.G., Plaxton, W.C., 1990. Purification and characterization of cytosolic aldolase from carrot storage roots. Biochem. J. 269, 133–139.

Moorhead, G.B.G., Plaxton, W.C., 1992. Evidence for an interaction between cytosolic aldolase and the ATP-and pyrophosphate-dependent phosphofructokinases in carrot storage roots. FEBS Lett. 313, 277–280.

Nishimura, M., Beevers, H., 1981. Isozymes of sugar phosphate metabolism in endosperm of germinating castor beans. Plant Physiol. (USA) 67, 1255–1258.

Ovádi, J., Srere, P.A., 2000. Macromolecular compartmentation and channeling. Int. Rev. Cytol. 192, 255–280.

Plaxton, W.C., 1996. The organization and regulation of plant glycolysis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 185–214.

Podestá, F.E., Plaxton, W.C., 1991. Kinetic and regulatory properties of cytosolic pyruvate kinase from germinaitng castor oil seeds. Biochem. J. 279, 495–501.

Pontremoli, S., Melloni, E., Salamino, F., Sparatore, B., Michetti, M., Singh, V.N., Horecker, B.L., 1979. Evidence for an interaction between fructose 1,6-bisphosphatase and fructose 1,6-bisphosphate aldolase. Arch. Biochem. Biophys. 203, 356–363.

Schnarrenberger, C., Krüger, I., 1986. Distinction between cytosol and chloroplast fructose-bisphosphate aldolases from pea, wheat, and corn leaves. Plant Physiol. (USA) 80, 301–304.

Schwab, W., Aharoni, A., Raab, T., Pérez, A.G., Sanz, C., 2001. Cytosolic aldolase is a ripening related enzyme in strawberry fruits (*Fragaria* × *ananassa*). Phytochemistry 56, 407–415.

Siegnthaler, P.A., Douet-Orhant, V.D., 1994. Relationship between the ATP content measured at three imbibition times and germination of onion seeds during storage at 3, 15 and 30 °C. J. Exp. Bot. 45, 1365–1371.